Interallelic Complementation Among DER/*flb* Alleles: Implications for the Mechanism of Signal Transduction by Receptor-Tyrosine Kinases

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ABSTRACT

The large number of available embryonic lethal alleles in the Drosophila EGF receptor homolog (DER)/*faint little ball* locus allowed us to test the possibility of positive or negative interactions among different DER alleles. These interactions were monitored by examining the embryonic cuticular phenotypes of different heteroallelic combinations. Several positive interactions were identified, while negative interactions were restricted to a single allele. This is the first example of positive interactions within the same cell type among alleles of a receptor tyrosine kinase gene. The basis for these interactions is likely to arise from the mechanism of signal transduction by receptor tyrosine kinases, which involves receptor aggregation. A combination of two different DER mutant proteins defective in temporally distinct stages of the signal transduction process, may thus form a functional heterodimer. The mutation sites in four alleles showing positive interactions were localized. They identify regions within the protein which are likely to be important for these temporally distinct signal transduction processes.

THE Drosophila EGF receptor homolog (DER) is a member of the receptor tyrosine kinase family (LIVNEH *et al.* 1985; SCHEJTER *et al.* 1986) and shows an equal degree of similarity to the vertebrate EGF receptor (ULLRICH *et al.* 1984) and neu proteins (BARGMANN, HUNG and WEINBERG 1986). DER was also shown to share functional properties with the vertebrate receptors: it possesses a tyrosine kinase activity which can be enhanced by a mutation in the transmembrane domain, in a manner similar to the oncogenic mutation in *neu* (WIDES, ZAK and SHILO 1990). The functional similarity indicates that important aspects of the mechanism of signal transduction are common to DER and its vertebrate homologs.

Characterization of the phenotypic consequences of mutations in the DER locus has demonstrated that the protein carries out a diverse set of roles during Drosophila development. The pleiotropic nature of DER function is in accordance with the wide range of tissues and developmental stages in which it is expressed (LEV, SHILO and KIMCHI 1985; SCHEJTER *et al.* 1986; KEMMERMEYER and WADSWORTH 1987; ZAK *et al.* 1990; KATZEN, KORNBERG and BISHOP 1991). Phenotypic and genetic analyses have clearly implicated DER as a participant in developmental mechanisms which are based on cell-cell interactions. The transmembrane receptor structure of the DER protein strongly suggests that DER is a mediator of these interactions. The dominant set of *Ellipse* mutations in the DER locus lead to the development of compound eyes with a significantly reduced number of ommatidia (BAKER and RUBIN 1989). This phenotype suggests that in the larval eye imaginal disc, DER is involved in the transmission of inhibitory signals between cells at the stage when the initial number and spacing of photoreceptor preclusters is determined (BANERJEE and ZIPURSKY 1990). Another set of mutations in the DER locus, *torpedo*, result in female sterility due to the production of ventralized embryos in ventralized egg shells (PRICE, CLIFFORD and SCHÜPBACH 1989; SCHÜPBACH 1987). Because the activity of DER in the ovary was shown to be required only in the somatic follicle cells, models for the determination of embryonic dorsoventral polarity place DER as the mediator in the transmission of polarity-determining signals from the oocyte to the follicle cells (MANSEAU and SCHÜPBACH 1989).

In contrast to the postembryonic functions of DER described above, more severe lesions in the locus result in embryonic lethality with a distinct phenotype termed *faint little ball* (*flb*) (NÜSSLEIN-VOLHARD, WIESCHAUS and KLUDING 1984; SCHEJTER and SHILO 1989; PRICE, CLIFFORD and SCHÜPBACH 1989; CLIFFORD and SCHÜPBACH 1990). Amorphic *flb* mutant embryos lack head structures, exhibit telson defects, fail to secrete ventral denticle bands and to retract their germ bands. As a result, the amorphic alleles give rise to a "curled" ball-like structure lacking ventral setae but retaining the typical dorsal hairs. Another aspect of the embryonic phenotype is the collapse of the scaffold of the embryonic central nervous

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system after its initial assembly (SCHEJTER and SHILO 1989; ZAK *et al.* 1990). The diversity of embryonic tissues affected in *flb* mutants complicates the elucidation of the basis for the embryonic phenotype.

The dramatic manifestations of the amorphic *flb* phenotype provide convenient landmarks by which alleles which give rise to a less severe phenotype can be functionally assayed. Indeed, the analysis of *flb* alleles shows that they constitute an allelic series, and a number of intermediate and weak alleles have been identified (SCHEJTER and SHILO 1989; CLIFFORD and SCHÜPBACH 1990). As a rule, in hypomorphic alleles all aspects of the embryonic phenotype display a reduced severity. For example, intermediate alleles develop patches of denticle bands and show partial germ band shortening, while weak alleles display telson material, some head structures, an almost fully retracted germ band and normal ventral cuticle.

Most DER proteins encoded by the large collection of EMS-induced *flb* alleles have retained residual biological activity. This was shown by observing biochemical activity in *flb* alleles (SCHEJTER and SHILO 1989; this work) and by the ability of some alleles to complement postembryonic DER functions (CLIFFORD and SCHÜPBACH 1990; BAKER and RUBIN 1989; our unpublished results). The fact that most *flb* alleles have preserved some functions of the protein, provides us with powerful tools to study defects in the signal transduction process mediated by DER. In principle, we view each of the point mutations as potentially affecting one or more of the protein-protein interactions mediated by this receptor (*e.g.*, recognition of ligands, aggregation of receptor molecules, and phosphorylation of cellular substrates).

Signal transduction by receptor tyrosine kinases is envisaged as a multistep process (reviewed in SCHLESSINGER 1988; ULLRICH and SCHLESSINGER 1990). Ligand binding induces a conformational change at the extracellular domain, which leads to oligomerization. The consequent juxtapositioning of the cytoplasmic kinase domains leads to tyrosine trans-phosphorylation of the C-terminal tails. The tyrosine-phosphorylated residues on the cytoplasmic domain of the kinase provide a cue for the association of substrates via their SH2 domains (SKOLNIK *et al.* 1991). The associated substrates are finally phosphorylated by the kinase domain on tyrosine residues. The intermolecular activation model is based on a variety of experiments carried out on different members of the receptor tyrosine kinase family, and appears to be universal for all receptor tyrosine kinases. For the EGF receptor, it has been shown that cross-linking of receptors enhances autophosphorylation, that dimers are formed from monomers following ligand addition, and that kinase negative mutants can be transphosphorylated by an EGF-stimulated wild-type receptor (YARDEN and SCHLESSINGER 1987a,b; HONEGGER *et al.* 1989).

The current model for receptor tyrosine kinase signal transduction leads to the expectation that receptors which are specifically defective in their capacity to phosphorylate the other receptor can be functionally complemented by mutant receptors in which this ability is intact but other aspects of the receptor function may be defective. In this work we concentrated on interactions among embryonic-lethal *flb* mutations, thus limiting ourselves to a single developmental stage. We therefore tend to consider the complementation phenomena described below as reflecting interaction between complementing alleles not only in the same stage of development, but also within the same cells. Therefore, they are likely to result from the inherent ability of the complementing alleles to restore defective interactions between receptors. We have identified both positive and negative interactions among the interallelic *flb* combinations tested. Localization of the mutation sites in the alleles involved in positive complementation allows us to define domains within the cytoplasmic region which appear to be important for temporally distinct stages in the signal transduction process, and are likely to affect recognition of different substrates.

MATERIALS AND METHODS

***Drosophila* stocks and procedures:** The generation of the ES *flb* alleles was previously described (SCHEJTER and SHILO 1989). *Df(2R)PK1* (57C5-57F5,6) and the JE alleles were provided by J. O'DONNEL and all of the other *flb* alleles used in this work were provided by C. NÜSSLEIN-VOLHARD and E. WIESCHAUS (NÜSSLEIN-VOLHARD, WIESCHAUS and KLUDING 1984).

The genetic crosses were set up in plastic tube blocks that were positioned on sugar/agar plates supplemented with yeast. Cuticle preparations were made by dechorionating unhatched embryos in bleach, mounting in 1:1 Hoyer's-lactic acid followed by overnight incubation at 60° (WIESCHAUS and NÜSSLEIN-VOLHARD 1986). For each cross, 100–200 embryonic cuticles were scored and classified. The degree of variability in phenotypes within the progeny of each cross was minimal.

Central nervous system (CNS) staining of embryos by anti-HRP antibodies was done as previously described (SCHEJTER and SHILO 1989).

DNA procedures: DNA was prepared from flies carrying an *flb* allele and the original parental chromosome, or from *flb* homozygous embryos selected under halocarbon oil. The DNA was prepared by homogenizing the flies in a buffered solution (0.1 M Tris-HCl, 0.1 M EDTA, 1% sodium dodecyl sulfate), incubating the homogenate at 70° for 30 min, and adding potassium acetate to 1.1 M followed by 30 min incubation at 0°. Protein and cellular debris were removed by centrifugation in a microfuge and the DNA was precipitated from the supernatant by adding 0.5 volume of isopropanol. The genomic DNA was then used for amplification in polymerase chian reaction (PCR) or digested with restriction enzymes for use in denaturing gradient gels.

The following PCR amplification protocol was used. Genomic DNA was added to a 50-ml reaction mixture contain-

ing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween 20, 0.1% NP-40, 0.8 mg/ml bovine serum albumin, 250 μM of each dNTP, 50 pmol of each primer and 2 units of Taq polymerase. The reaction mix was overlayed with paraffin oil and was cycled 15 times between 94° 45 sec, 52° 60 sec, 72° 5 min and 20 times between 94° 45 sec, 61° 90 sec, 72° 5 min. The region common to both splicing alternatives that contains most of the coding region of DER (SCHEJTER and SHILO 1989) was divided into two parts that were separately amplified. A primer whose 3′ end is 52 bases upstream of the first common exon was used in combination with a primer whose 3′ end is 2716 bases away to amplify the N-terminal region (5′ GCTGAG **CTCGAGCCATTAGCCCGCATCGACAC** 3′ and 5′ **AGAGCCGTCGACATCTTGACTG TTTCCTT-CTTGGC** 3′, respectively). Bold-face letters show the nucleotides complementary to the DER sequence). A primer whose 3′ is 6 bp downstream of the termination codon was used in combination with a primer whose 3′ end is 2023 bases upstream to amplify the C-terminal region (5′ **CTATGGGTCG**ACC**TAGGCTCTGTACAGGCGCAC** 3′ and 5′ **GAGCA GCTCGAGTGCTTCCAGCGCCACC** 3′, respectively). The oligonucleotides contain sequences homologous to the DER sequence flanked by about 12 bases containing restriction enzyme sites.

The localization of mutations was done using the denaturing gradient gel procedure (MAYERS, MANIATIS and LERMAN 1987). Briefly, genomic DNA samples from flies heterozygous for *flb* alleles were digested with restriction enzymes and run on a 20–80% formamide-urea gradient acrylamide gel at 60°, transferred to Hybond-N filters (Amersham) and hybridized with a DER DNA probe. In cases were a band shift was detected, indicating that a mutation resides in that fragment, DER genomic DNA was amplified by PCR, cut with the same restriction enzyme and run on the gradient under similar conditions. The shifted band could then be visualized by ethidium bromide staining, cut out of the gel and used as a probe on Southern blots of DER DNA digested with several enzymes and run on agarose gels. After the location of the shifted fragment was determined, the mutant DNA region was sequenced. Sequencing was done on single-stranded DNA obtained directly from the PCR (GYLLENSTEN and ERLICH 1988) (in the case of the *1F26*, *2C82*, *2L65* and *2W74* alleles), or from a pool of several independent plasmid clones containing PCRed DNA prepared from homozygous mutant embryos (in the case of the *2X51* allele). A control sample containing the DNA of the parental chromosome of each set of alleles was used throughout the analysis of the mutation sites (the gradient gels, cloning and sequencing). Thus, any naturally occurring polymorphism between fly stocks was excluded.

***In vitro* kinase assays:** *In vitro* kinase assays of DER in embryo extracts were carried out as previously described by Schejter and Shilo (1989). Briefly, 30 embryos homozygous for *flb* mutations were selected on the basis of their phenotype, and lysed in 100 μl buffer containing 20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 μg/ml benzamidine, 1 μg/ml leupeptin and 1 mM PMSF. The reaction was started by adding 5 μl [γ-^{32}P]ATP (10 mCi/ml, 3000 Ci/mmol) and 2 μl of 1 M $MnCl_2$ and was allowed to proceed 10 min on ice. The reaction was terminated by adding 33 μl of termination mixture (23 μl of 200 mM Na_2HPO_4, pH 9.0, 9 μl of 500 mM EDTA and 1 μl of 100 mM ribsomal ATP), and the DER protein was immunoprecipitated with anti-DER antibodies in the presence of 0.2 mM Na_3VO_4. The proteins were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and autophosphorylated DER was visualized by autoradiography.

RESULTS

Most DER/*flb* alleles retain some biological or biochemical activity: Following EMS treatment, the mutation frequency in the locus encoding DER is three- to fivefold higher than in other loci (NÜSSLEIN-VOLHARD, WIESCHAUS and KLUDING 1984; SCHEJTER and SHILO 1989). This high frequency can be partially accounted for by the size of the encoded protein (over 1400 amino acids). It is also likely to result from the high degree of structural conservation of DER and the requirement to maintain its interactions with multiple proteins such as the ligand(s) substrate(s) and DER itself. The activity of over 20 alleles of DER/*flb* was assayed by a variety of methods, including: (1) The severity of the cuticle phenotype (SCHEJTER and SHILO 1989; CLIFFORD and SCHÜPBACH 1990); (2) *in vitro* tyrosine autophosphorylation activity (SCHEJTER and SHILO 1989 and this work); and (3) the ability of embryonic lethal *flb* alleles to complement larval or adult functions of DER (CLIFFORD and SCHÜPBACH 1990). A convenient assay is based on the observation of BAKER and RUBIN (1989) that the *Ellipse* phenotype depends on the presence of an active DER gene on the other chromosome. Several *flb* alleles that have a severe embryonic phenotype (such as *2L65*, *2X51* and *3B92*), nevertheless retain the ability to display a rough-eye phenotype in conjunction with *Ellipse*.

The conclusion from the three assays is that only three alleles behave as complete nulls by all assays (flb^{IK35}, flb^{IPO2} and flb^{3B41}). The majority of alleles are thus likely to represent missense mutations that are defective only in some aspects of the function of DER. The collection of DER/*flb* alleles provides an excellent starting point to identify interactions (positive or negative) among different alleles.

Interactions between DER mutant alleles: In order to identify interactions between DER embryonic lethal alleles, we generated a matrix of crosses between flies heterozygous for different mutant alleles, and examined the embryonic cuticular phenotype of the resulting heteroallelic offspring (Figure 1). The relative uniformity of the phenotypic defects observed in embryos homozygous or hemizygous for any one allele, and the variety of structures affected by mutations in the DER/*flb* gene, make the *flb* cuticular phenotype a sensitive biological marker for the severity of each allele. A specific allele combination was defined as showing positive complementation if the heteroallelic phenotype was milder than that displayed by the weaker of the two mutations in the homozygous state. Conversely, a heteroallelic combination was defined as interacting negatively if the resulting phenotype was more severe than the hemizygous state of the weaker allele in the pair.

	Df	*1F26*	*1K35*	*1PO2*	*2C82*	*2EO7*	*2G31*	*2L65*	*2W74*	*2X51*	*3B41*	*3B92*	*3C81*	*3C87*	*ES3*	*ES45*	*JE1*	*JE3*	*JE13*	*SH2*
Df	S	I/W	S	S	S/I	I	S	S	I	S(I)	S	S	S	S	I	I	S	W	S	I
1F26	I/W	W	I/W	W	W	W	S(I)	W	W	S/I	I/W	I/W	W	I/W	W	I/W	I/W	W	I/W	I/W
1K35	S	I/W	S		S/I	I	S	S	I	S	S	S	S			I	S			I
1PO2	S	W		S	S/I		S	S	I/W	S	S	S	S				S	I	S	I/W
2C82	S/I	W	S/I	S/I	I	I/W	S/I	S/I	**WT/W**	**WT/W**	S/I	I	S/I	I/W	W	**WT/W**	I	W	I	I/W
2EO7	I	I/W	I		I/W	W	I	I/W	I/W	I	I	I/W	I	I	W	W	I/W	W	I/W	I/W
2G31	S	S(I)	S	S	S/I	I	S	S	I	S(I)	S	S	S	S	I/W	I	S	I/W	S	I/W
2L65	S	W	S	S	S/I	I/W	S	S	**WT/W**	**WT/W**	S	S	S	S/I	I/W	**WT/W**	S	W	S	I/W
2W74	I	W	I	I/W	**WT/W**	W	I	**WT/W**	W/I	I/W	I	I	I	W	W	W/I	I/W	W	I/W	I/W
2X51	S(I)	S/I	S	S	**WT/W**	I	S(I)	**WT/W**	I/W	S(I)	S(I)	S(I)	S(I)	S(I)	I/W	I	S(I)	W	S(I)	I/W
3B41	S	I/W	S	S	S/I	I	S	S	I	S(I)	S	S	S	S	I	I	S	I	S	I
3B92	S	I/W	S	S	I	I/W	S	S	I	S(I)	S	S	S	S	I/W	I/W	S	W	S	I
3C81	S	W	S	S	S/I	I	S	S	I	S(I)	S	S	S	S	I/W	I	S	I	S	I
3C87	S	I/W			I/W	I	S	S/I	W	S(I)	S	S	S	S	I	I	S	W	S	I
ES3	I	W			W	W	I/W	I/W	W	I/W	I	I/W	I/W	I	W	W	I		I	I/W
ES45	I	I/W	I		**WT/W**	W	I	**WT/W**	I/W	I	I	I/W	I	I	W	I	I	W	I	I/W
JE1	S	I/W	S	S	I	I/W	S	S	I/W	S(I)	S	S	S	S	I	I	S	I/W	S	I/W
JE3	W	W		I	W	W	I/W	W	W	I	I	W	I	W		W	I/W	W	W	W
JE13	S	I/W		S	I	I/W	S	S	I/W	S(I)	S	S	S	S	I	I	S	W	S	W
SH2	I	I/W	I	I/W	I/W	I/W	I/W	I/W	I/W	I/W	I	I	I	I	I/W	I/W	I/W	W	W	I/W

FIGURE 1.—The cuticle phenotype of interallelic *flb* combinations. Cuticle preparations of embryos bearing various *flb* allele combinations were analyzed and classified into five phenotypic groups. S = a severe *flb* cuticular phenotype that shows complete deterioration of the head, no germ-band retraction and no ventral denticle bands. I = an intermediate *flb* cuticular phenotype in which some head structures are formed, the germ-band is partially shortened and the denticle bands are formed as small patches on the ventral side. W = a weak *flb* cuticular phenotype showing less severe head defects, an almost fully retracted germ band and an almost normal ventral cuticle. S(I) shows a severe phenotype with some denticle patches. S/I and I/W display a phenotype between severe and intermediate and between intermediate and weak, respectively. The first row and the first column specify the alleles tested in this matrix, where *Df* is *Df(2L)PK1*, a deficiency encompassing the DER locus. Combinations showing negative complementation are shown as shadowed boxes with empty letters, while positive complementations rescuing the cuticle phenotype are marked by bold letters as WT/W (wild type/weak) in shadowed boxes.

We examined 161 heteroallelic combinations resulting from crosses between 19 *flb* alleles. Overall, positive or negative interactions appear to be the exception rather than the rule (Figure 1, shadowed rectangles). Positive interactions were more prevalent than negative ones, and several alleles participate in them. Out of all heteroallelic combinations examined in this work, we concentrated only on the positive complementations that were clear-cut, resulting in a substantial improvement of the cuticular phenotype. In these cases, severe or intermediate allele combinations show only extremely mild cuticular defects compared with the expected *flb* phenotype. These interactions were restricted to five alleles (Figure 1, bold letters). Analysis of the pattern of positive complementations demonstrates that the interacting alleles can be divided into two classes. The first group includes alleles *2C82* and *2L65*, while the other includes the *2X51*, *ES45* and *2W74* alleles. No interactions are observed within either class of alleles, while all intergroup combinations display complementation. Negative interactions appear to be restricted to a single allele (*JE3*) that induces the phenomenon when combined with a variety of other alleles (Figure 1, open letters). These negative interactions were not followed in detail, but we assume that the receptor encoded by the *JE3* allele is more "compatible" with itself in processes of aggregation or *trans*-activation than with other mutant receptors. The formation of heterodimers when *JE3* is crossed to other alleles appears to reduce its biological activity.

We have concentrated our studies on a detailed examination of the positive interactions. Figure 2 shows the phenotypic complementation between representative alleles of each group. The alleles *2X51* and *2L65*, which exhibit characteristically severe *flb* defects as homozygotes (Figure 2, b and c), complement each other to produce an extremely improved cuticular phenotype (Figure 2e). The heteroallelic embryos undergo germ-band retraction, secrete a normal pattern of ventral cuticle, and show only minor head defects (mainly dislocation and fusion of the anterior mouth parts). In other heteroallelic combinations, milder embryonic phenotypes can also be improved compared to the homozygous phenotypes (*e.g.*, the interaction between *2C82* and *2X51* as shown in Figure 2f). No interaction is observed between alleles belonging to the same class (*e.g.*, *2L65* and *2C82* in Figure 2g). We note that the two complementation groups include alleles exhibiting different severity grades of the embryonic phenotype when homozygous, and interpret this observation as indicating that the positive interactions result from qualitative complementation of distinct functions lost in alleles of one group and retained by the other.

To determine whether the complementation observed at the cuticular level extends also to other aspects of the flb embryonic phenotype, the morphol-

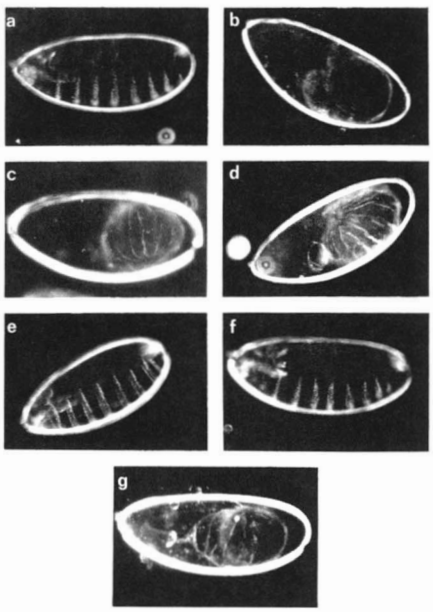

FIGURE 2.—Embryonic cuticle morphology resulting from *flb* interallelic complementation. The wild type embryos (a) exhibit the characteristic denticle bands at the ventral side and a fully retracted germ band, while the homozygous *flb* mutants of both classes (b) flb^{2X51}/flb^{2X51}, (c) flb^{2L65}/flb^{2L65} and (d) flb^{2C82}/flb^{2C82} show no, or severely reduced, denticles together with head and germ-band retraction defects. Interclass allele combinations show positive complementation, resulting in a cuticle similar to wild type (e) flb^{2X51}/flb^{2L65} and (f) flb^{2X51}/flb^{2C82}. In contrast, intragroup combination show no interaction (g) flb^{2L65}/flb^{2C82}.

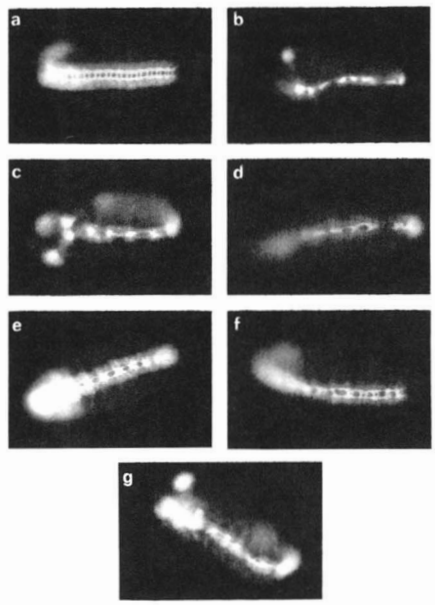

FIGURE 3.—Embryonic CNS morphology resulting from *flb* interallelic complementation. The CNS phenotypes of embryos resulting from both inter- and intraclass crosses were monitored by anti-HRP staining. The wild-type embryos (a) exhibit the characteristic arrangement of longitudinal and horizontal axon tracks. Homozygous *flb* mutants of both classes (b) flb^{2X51}/flb^{2X51}, (c) flb^{2L65}/flb^{2L65} and (d) flb^{2C82}/flb^{2C82} show fusion of the scaffold of the central nervous system along the longitudinal axon tracks and among the horizontal commissures, and discontinuities along its longitudinal axis. Interclass allele combinations show a positive complementation leaving only mild defects in the commissures (e) flb^{2X51}/flb^{2L65} and (f) flb^{2X51}/flb^{2C82}. In contrast, intragroup combination show no interaction (g) flb^{2L65}/flb^{2C82}.

ogy of the CNS of several allelic combinations was examined. As seen in Figure 3, b–d, embryos homozygous for severe or intermediate *flb* mutations exhibit fusion of the scaffold of the central nervous system along the longitudinal axon tracks and among the horizontal commissures, and discontinuities along its longitudinal axis. The mutant CNS assumes a twisted shape, and misrouting of segmental and intersegmental nerves can be observed. In contrast, the combination of two complementing alleles rescues the severe CNS phenotype, leaving only relatively minor defects (Figure 3, e and f). In the rescued embryo, longitudinal axon tracks are separated and continuous, and the normal number of neuromeres is observed. However, some defects are still obvious in the horizontal commissures, which are often fused. The mild defects in the head and in the CNS observed in the complementing crosses may explain why these embryos do not develop further.

Localizing the mutation sites in interacting *flb* alleles: To understand the mechanism of the observed interactions, we characterized the molecular lesions in mutant DER alleles from the complementing classes. The regions in which mutations reside were localized using denaturing gradient gel electrophoresis (MAYERS, MANIATIS and LERMAN 1987). For each of the alleles described below, only a single fragment from the entire DER gene showed an altered mobility in the gradient gels, and was chosen for further analysis. The mutation site was then determined by direct sequencing of DNA amplified by the PCR (GYLLENSTEN and ERLICH 1988), or by sequencing a mixture of several independent plasmid clones obtained from PCR-derived mutant DNA. The migration and sequence of the mutant alleles was always compared to the parental chromosome or to other alleles derived from the same screen, thus excluding the possibility of identifying naturally occurring polymorphisms.

Mutations of one class (*2L65* and *2C82*) were localized to the C-terminal end of the kinase domain (subdomains X and XI in HANKS, QUINN and HUNTER 1988) (Figure 4). This region contains some highly conserved residues common to all classes of tyrosine kinases. Functionally, the X-XI stretch has been shown to be crucial for kinase activity, because its truncation abolishes the biological activity of v-*src* (HANKS, QUINN and HUNTER 1988 and references therein), yet no defined function has been assigned to it.

The two mutations we identified within subdomains X-XI are only 11 amino acids apart. The *2C82* mutation leads to substitution of glycine-1106 by a serine residue (Figure 4). This glycine residue is conserved in most of the tyrosine kinases (HANKS, QUINN and

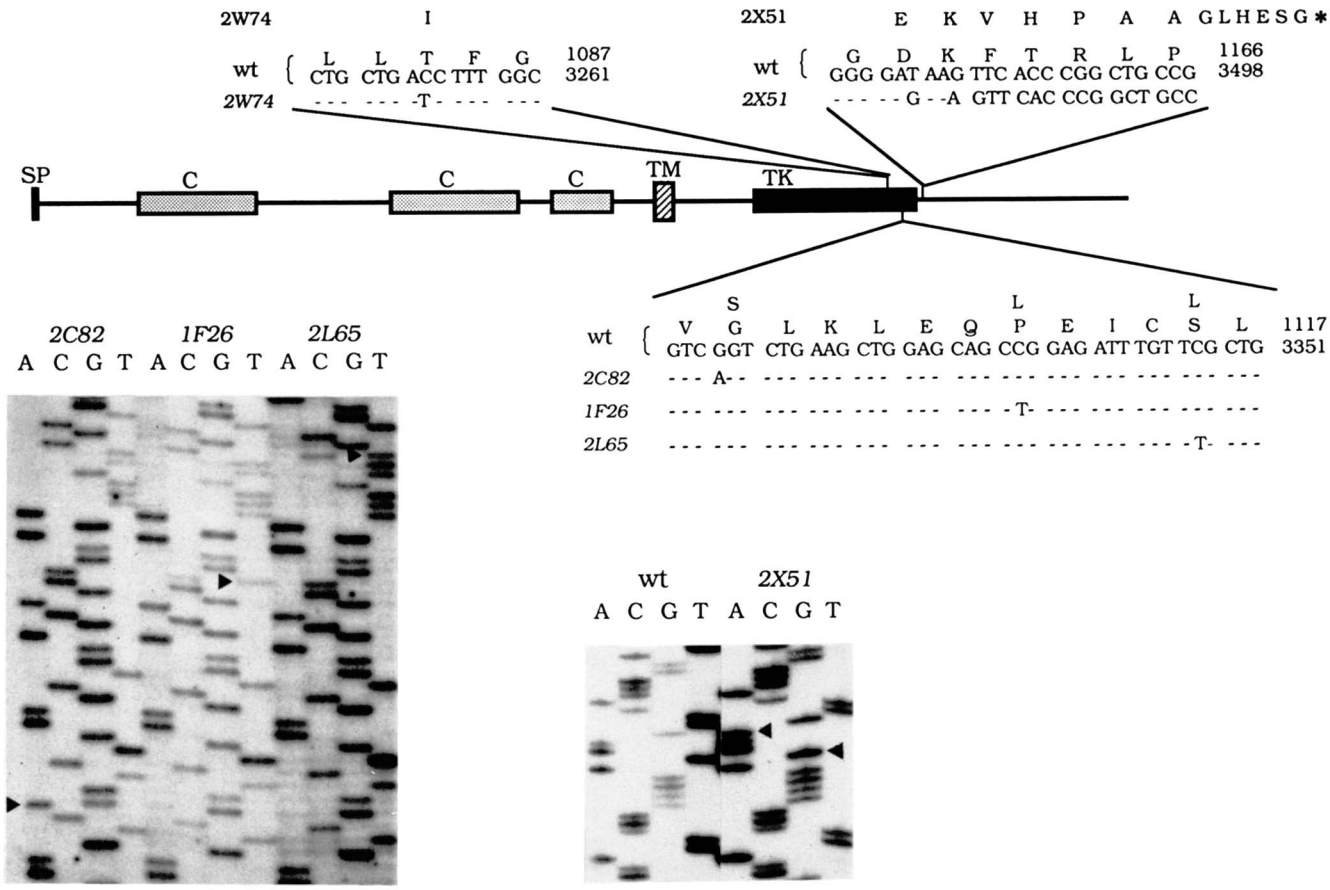

FIGURE 4.—Mutation sites of interacting *flb* alleles. For each mutation the altered amino acids are shown above the wild-type amino acid sequence, and the corresponding nucleotide transition below the wild-type nucleotide sequence. Sequencing gels displaying the mutation sites are shown at the bottom. Sequencing of the *2W74*, *2C82*, *1F26* and *2L65* alleles was done on single-stranded DNA from heterozygous flies, while the *2X51* allele sequence was done on a pool of several independent plasmid clones containing PCRed DNA prepared from homozygous mutant embryos (see MATERIALS AND METHODS). Abbreviations: SP, signal peptide; TM, transmembrane domain; C, cysteine rich domains; TK, tyrosine kinase region. The amino acid and nucleotide numbering relates to the type II 5′ splicing alternative (SCHEJTER *et al.* 1986).

HUNTER 1988). It was also shown to be important for the transforming activity of the v-*src* tyrosine kinase, because the protein mutated at this position (substitution of glycine-478 by aspartic acid) is temperature-sensitive (FINCHEM and WYKE 1986). The *2L65* mutation results in a substitution of serine-1116 by leucine (Figure 4). The serine residue changed by the *2L65* mutation is conserved in the *Caenorhabditis elegans* EGF receptor homolog *let-23* (AROIAN *et al.* 1990), while the EGF receptor, the neu receptor and the newly identified HER3 receptor show a conservative amino acid substitution (threonine) at this position (Figure 5) (HANKS, QUINN and HUNTER 1988; KRAUS *et al.* 1989; PLOWMAN *et al.* 1990).

Searching for the mutation sites in alleles of the second complementation group, we found that the *2X51* allele carries two closely linked mutations. The first changes aspartic acid to glutamic acid at position 1160, while the second is a one-base insertion that changes the reading frame at amino acid position 1161, leading to termination of translation only 12 amino acids further downstream (Figure 4). The *2X51* mutation thus represents a truncation of the entire C-terminal tail, while the kinase domain is left intact. The severe *flb* phenotype resulting from this structural alteration demonstrates that the C-terminal tail is functionally essential. Recent experiments with molecules of the EGF receptor and neu in which most of the cytoplasmic tail has been deleted, demonstrate that the tail is crucial for the biological activity of the receptors and for their ability to efficiently phosphorylate exogenous substrates (DIFIORE *et al.* 1990). Exchanging the C-terminal tails between the two receptors resulted in an alteration of the activity level of the respective receptors, suggesting that this domain is important for the function and regulation of the receptor. The *2W74* mutation was identified as a substitution of threonine 1087 by isoleucine. This residue is conserved in all members of the EGF receptor class (Figure 5), as well as in several other tyrosine kinases. It is interesting to note that this mutation falls in subdomain IX of the kinase, upstream of the se-

		2W74	2C82	1F26	2L65	
		I	S	L	L	
		↑	↑	↑	↑	
DER	(1083)	LLTFGQRPHEN	IPAKDIPDLIEVGLKLEQPEI		CSLD	(1118)
EGFR	(907)	-M---SK-YDG	---SE-SSIL-K-ER-P--P-		-TI-	(942)
neu	(920)	-M---AK-YDG	---RE----L-K-ER-P--P-		-TI-	(955)
HER3	(885)	-M---AE-YAG	LRLAEV---L-K-ER-A--Q-		-TI-	(920)
c-src	(455)	-T-K-RV-YPG	MVNREVL-QV-R-YRMPC-PE		-PES	(490)
let-23	(1080)	II----S-YQG	MSTDS-HNFLKD-NR-S--PN		--Q-	(1115)
Subdomain		**IX**	**X**		**XI**	

FIGURE 5.—Amino acid sequence alignment of different tyrosine kinases in the mutated region. The amino acid sequence of DER is compared with that of the EGF receptor, neu, HER3, let-23 and src. The amino acid sequence alteration in *flb* alleles is shown at the top row. Identical residues are denoted with dashes. For the receptors, the amino acid numbering includes the signal peptide.

quence that is truncated by the *2X51* mutation belonging to the same class. The possible functional similarities between these two mutations will be discussed. Due to technical problems, the *ES45* mutation was not localized.

Alleles participating in the positive interactions retain *in vitro* kinase activity: The intermolecular phosphorylation occurring between aggregated receptors suggests that tyrosine kinase activity is a crucial step in trans-activation. We therefore asked whether alleles displaying positive complementation retain kinase activity. An *in vitro* kinase assay of protein extracts derived from embryos homozygous for the mutations of interest represents the most sensitive approach to address this issue. The assay is carried out on detergent-solubilized lysates and thus monitors the intramolecular, ligand independent autophosphorylating activity of the receptor (WIDES, ZAC and SHILO 1990).

The *in vitro* kinase assay was performed on protein extracts from embryos homozygous for mutations participating in the positive interactions (Figure 6). These were collected after 7–14 hr of development, based on identification of the first manifestations of the *flb* phenotype. The *2L65* and *ES45* proteins showed autophosphorylation of DER that was comparable to that for wild-type embryos. The activities of *2X51* (SCHEJTER and SHILO 1989), *2C82*, and *2W74* homozygous embryos (Figure 6) did not exceed the background level defined by control embryos homozygous for the *PK1* deficiency that removes the gene for DER. While the inactivity of the *2X51* protein was expected (because it lacks the entire C-terminal tail containing both the target sites for autophosphorylation and the epitopes recognized by our antibodies), it is surprising that both *2C82* and *2W74* are inactive. A possible explanation for these results will be discussed below.

DISCUSSION

In this work we utilized the large number of *flb* alleles giving rise to an embryonic lethal phenotype,

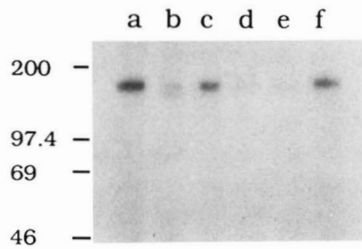

FIGURE 6.—*In vitro* kinase activity of *flb* mutant proteins. An *in vitro* kinase assay was performed on protein extracts from 7–14-hr *flb* mutant embryos, followed by immunopercipitation with anti DER antibodies. Lanes: a, wild-type embryo extracts; b, *Df(2L)PK1/Df(2L)PK1*; c, flb^{2L65}/flb^{2L65}; d, flb^{2C82}/flb^{2C82}; e, flb^{2W74}/flb^{2W74}; f, flb^{ES45}/flb^{ES45}. Molecular weight (×1000) markers are shown on the left. Note: the faint band observed in lanes d and e does not correspond to the DER protein, because it is found in extracts of *Df(2R)PK1* homozygous embryos that can be unambiguously picked (lane b); it is also observed following immunopercipitation with nonimmune rabbit serum (SCHEJTER and SHILO 1989).

to look for genetic interactions among them. Because these alleles were isolated following EMS mutagenesis, most are likely to represent missense mutations. Indeed, when the different alleles were scored for residual biological or biochemical activity, most showed some activity, indicating that they are hypomorphs. Although the embryonic phenotype is complex and involves several independent effects, each of the hypomorphic alleles exhibits the full spectrum of phenotypic effects. In other words, in each of the alleles, all aspects of the phenotype were affected to the same extent. Our inability to genetically separate between the different facets of the embryonic phenotype suggests that the same signal transduction pathway [*i.e.*, ligand(s) and substrate(s)] is employed in the diverse biological pathways mediated by DER, including the differentiation of head structures, ventral epidermis and CNS. This result is strikingly different from the situation with the *C. elegans* EGF receptor homolog *let-23* in which different mutations independently affect the various aspects of its null phenotype, pointing to tissue-specific functions of this receptor (AROIAN and STERNBERG 1991).

Genetic assays for the interaction of transmembrane proteins have been successfully employed in the past in different biological systems. The Drosophila *Notch* locus and the *C. elegans lin12* locus encode proteins that are believed to aggregate in the course of signal transduction, and contain multiple EGF repeats (WHARTON *et al.* 1985; GREENWALD 1985). Positive and negative interactions between *Abruptex* alleles of the *Notch* locus and also between *lin12* alleles were indeed observed (FOSTER 1975; GREENWALD and SEYDOUX 1990).

Receptor tyrosine kinases are expected to be prone to positive and negative interaction among mutant alleles, because their mechanism of signal transduction is postulated to involve an aggregation step in which

the cytoplasmic domains *trans*-activate each other. This paper describes a genetic complementation scheme among different alleles in the DER/*flb* locus. The motivation for performing this screen was to identify discrete defects within DER that could be complemented by alleles carrying aberrations in other aspects of the function of the receptor. These mutants may give a clue to the process of communication between receptors, which is an essential component of the signal transduction cascade.

The matrix identified positive interactions among alleles in a restricted set of combinations. This feature was seen for five alleles and in six out of 161 combinations that were tested. The complementing mutations defined two classes, containing two and three alleles each. The complementation encompassed all aspects of the *faint little ball* embryonic phenotype. The cuticle phenotype appears wild type with respect to the retraction of the germ band and the secretion of ventral denticle bands and shows minor head defects compared with those seen in *flb* embryos. The CNS phenotype also showed considerable improvement, but defects in the horizontal commissures could still be identified. The incomplete rescue of the head and the CNS phenotype may indicate that they are quantitatively more sensitive than other aspects of the embryonic phenotype.

In addition to the rescue of all aspects of the phenotype, it is important to emphasize that the division of the complementing alleles to the two classes does not correlate with the severity of the phenotype they display individually, and that a combination of two severe alleles such as *2L65* and *2X51* gives complete phenotypic rescue. This observation indicates that the complementation we observed cannot be explained by simple additive contributions of the complementing alleles. Rather, each class of alleles appears to be defective in a distinct subfunction of the receptor, leading to complete complementation by combination of the two classes.

In the *2L65* and *2C82* alleles, it was gratifying to find that the mutations are in close proximity, only 11 residues apart. Because the functional similarity between them stems from a structural similarity, they are likely to affect a subdomain of DER responsible for a defined function. Interestingly, another *flb* mutation, *1F26*, was also localized to this small interval (Figure 4). *1F26* is a temperature-sensitive mutation showing a weak phenotype at 18° and a severe *flb* phenotype at 29°. This mutation changes a highly conserved proline 1112 to leucine. In spite of the localization of the *1F26* lesion to the same region, this allele does not behave in the complementation assay as its neighboring mutations when tested at the restrictive temperature. We attribute this discrepancy to the dramatic sequence alteration in *1F26*, resulting from substitution of a conserved proline residue. Proline is known to have a pronounced influence on polypeptide secondary conformation by causing a bend and interrupting an α-helical structure. It is thus possible that such a change will alter the structure of the kinase domain in a more global way. Defects in more than just one function could thus prevent the *1F26* allele from being complemented by alleles of the *2X51* class. The localization of mutations belonging to the *2X51* class is less restricted. The *2X51* allele lacks the entire C-terminal tail. The *2W74* mutation is located within subdomain IX of the kinase, upstream to the sequence truncated in *2X51*.

The starting point for models that can be proposed to explain our complementation results is the speculation about the nature of the specific defects in the alleles of each class, and their effect on the function of the receptor. Two different concepts can be put forward. One possibility is that the defects caused by the complementing mutations are not affecting the interaction between receptors, but rather the range of substrates recognized by the kinase domain, or the spectrum of tissues in which each class of mutations is preferentially defective. The proper combination of alleles restores the full range of substrate recognition or the activity in all tissues. We view this possibility as unlikely because all alleles tested lead to the entire spectrum of the embryonic *flb* defects. Likewise, complementation of all embryonic defects is observed in the proper crosses, and even combinations of severe alleles completely restores the wild-type cuticular phenotype. To account for these observations by proposing an overlapping range of substrate specificity, one would have to assume that all tissues require exactly the same combination of substrates, and that failure to phosphorylate a single substrate can give rise to an amorphic phenotype. In addition, the localization of the *2C82* mutation to a residue conserved in all tyrosine kinases (including the nonreceptor class) suggests that we are dealing with a function that is common to all tyrosine kinases rather than with the determination of substrate specificity which is likely to be different for each of the members.

We propose the following model to account for the interallelic complementation results we observed. Because the aggregation and *trans*-activation of receptor tyrosine kinases is a multistep process, we assume that each of the two groups of alleles is defective in a distinct stage in the signal transduction process. A combination of the two groups, which affect *temporally distinct stages*, restores biological activity. One way to envisage the interaction is to assume that domains X-XI, which are defective in the *2L65* mutation class, participate in the *trans*-activation process. In order to *trans*-activate the partner kinase, a receptor must have both an intact domain X-XI and kinase activity. The

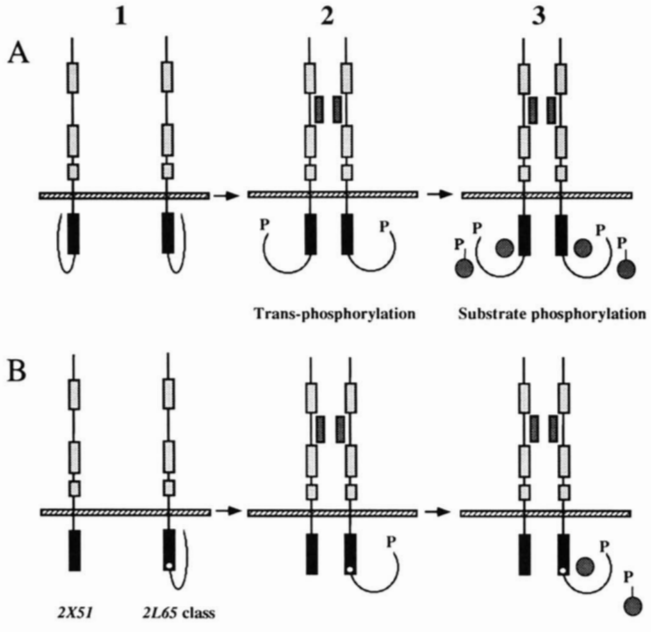

FIGURE 7.—A model for interallelic complementation of *flb* mutations. Signal transduction by the receptor is initiated by ligand binding, leading to aggregation and transphosphorylation (A2). The activated receptors are then capable of recognizing and phosphorylating cellular substrates, thus transmitting the signal intracellularly (A3). In the case of complementation between the flb^{2X51} and the flb^{2L65} classes, the flb^{2L65} group could be defective in the transphosphorylation process (B2). Conversely, the flb^{2X51} class could be defective in its ability to recognize and phosphorylate exogenous substrates (B3). In embryos carrying one *flb* allele of each class, the heterodimers formed after ligand binding are partially functional. The flb^{2X51} protein is capable of transphosphorylating the flb^{2L65} protein. Once phosphorylated, the flb^{2L65} protein is then able to associate with and phosphorylate exogenous substrates, thus restoring biological activity.

2X51 receptor with its tail deletion is thus capable of mediating this process. The 2L65 molecule, after being properly activated by the 2X51 receptor, can now associate with and phosphorylate exogenous substrates, because both its kinase and tail domains are intact. Biological activity of the DER pathway is thus restored. This model is schematically drawn in Figure 7B. The assignment of the interaction with substrates to the C-terminal domain that is deleted in *2X51* is corroborated by biochemical experiments showing the importance of the tyrosine-phosphorylated tail of the EGF receptor for association with substrate proteins such as GAP and PLC-γ (MARGOLIS *et al.* 1990). Although *2W74* was shown to behave as a member of the *2X51* class in the complementation tests, the *2W74* mutation was found to be located upstream to the region deleted in *2X51*. This result may be explained if we assume that the *2W74* mutation alters the ability of the kinase to recognize exogenous substrates, without altering its ability to recognize the tail of the partner receptor. Thus *2X51* and *2W74* may be defective in a similar process involving the association with or recognition of the exogenous substrates. It is interesting that mutations belonging to the two complementation classes fall in close proximity within the kinase domain (*2W74* being located only 21 residues upstream of *2C82* and 31 residues upstream of *2L65*). We assume that these mutations leave the kinase activity intact but alter its substrate specificity. It appears that the ability to recognize the tail can be altered without affecting the ability to recognize exogenous substrates and vice versa.

It has been shown that some of the DER/*flb* alleles participating in positive complementation retain kinase activity. Tyrosine kinase catalytic activity thus appears to be required not only for phosphorylation of exogenous substrates but also for the *trans*-activation process that is intimately linked to transphosphorylation of tyrosine residues on the C-terminal tail. It was puzzling to find that the *2C82* and *2W74* alleles did not show *in vitro* kinase activity. In all systems the catalytic activity of receptor tyrosine kinases was shown to be an essential requirement for their biological activity. It is thus difficult to envisage DER proteins with no kinase activity as giving rise only to an intermediate phenotype, or being able to complement each other as is the case with *2C82* and *2W74*. We do not have a definite answer, but suggest that the *in vitro* kinase assay is performed under conditions that are different from the *in vivo* situation, and may provide an altered milieu for the recognition of the C-terminal tail. Furthermore, phosphorylation *in vitro* is an intramolecular process, whereas the autophosphorylation of receptor tyrosine kinase *in vivo* is intermolecular (YARDEN and SCHLESSINGER 1987b). Several mutant proteins that may have a partial kinase activity *in vivo* could thus score as negative *in vitro*.

The experiments with interallelic combinations of the mouse *White spotting* allele (the c-*kit* receptor tyrosine kinase gene) also support the notion that formation of productive dimers requires that both receptors possess an active kinase domain. Alleles coding for a c-*kit* molecule with a defective kinase domain had an inhibitory effect when combined with the wild-type allele (NOCKA *et al.* 1990; TAN *et al.* 1990). Quantitatively, however, the c-*kit* locus appears to represent a special situation in which the normal level of activity of the kinase is very close to the threshold of required activity. Thus, even mice that carry a wild-type allele over a deficiency show a weak phenotype. The situation with DER is likely to be different than that of c-*kit* in that the normal activity of the receptor is in excess with respect to the threshold required. Flies heterozygous for a deficiency of DER show no trace of the mutant phenotype. In addition, severe alleles that have an inactive kinase as measured by the *in vitro* kinase assay (SCHEJTER and SHILO 1989) show no detectable phenotype in combination with the wild-type allele.

The inhibitory interaction we identified in our screen represents a different phenomenon than that observed in *White spotting* mice. It is restricted to a single allele (*JE3*) that appears to have negative interaction with a variety of other alleles. To account for this interaction we propose that JE3 dimers are more compatible with themselves than in heterodimers with receptors encoded by other alleles. Future identification of the *JE3* mutation site may reveal more about the nature of this compatibility. A similar situation is observed with the *Abruptex* alleles of the *Drosophila Notch* locus, where negative interaction between alleles appears to result from their incompatibility to function as dimers (FOSTER 1975).

In summary, our ability to detect positive interactions between several alleles of DER is consistent with and supports the model for receptor tyrosine kinase signal transduction, based on aggregation and *trans*-activation. Utilizing a combined genetic and molecular approach, we have identified regions of the DER receptor tyrosine kinase which are likely to be involved in discrete aspects of the signal transduction mechanism. One class appears to be unable to recognize the C-terminal tail as a substrate, while the other may impaired in its ability to recognize the proper exogenous substrates.

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